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(54) Title: IMPROVED TRANSAMINASE BIOTRANSFORMATION PROCESS

(57) Abstract

A process for making natural and unnatural amino acids which comprises reacting a first amino acid, a keto acid and a transaminase enzyme under conditions appropriate to produce a second amino acid and pyruvate; reacting the pyruvate with acetolactate synthase under conditions appropriate to produce a compound that does not react with the transaminase enzyme and separating the second amino acid.

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TITLE

IMPROVED TRANSAMINASE BIOTRANSFORMATION PROCESS

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to a process for producing amino acids employing aminotransferases. More particularly,

- 5 this invention relates to a process for making unnatural amino acids such as L-2-aminobutyrate and L-tert-leucine employing an aminotransferase.

Related Background Art

10

The process of the invention improves on prior art processes for making natural and unnatural amino acids using transaminase enzymes. Transaminases have been known in the literature for many years. See,

- 15 Transaminases, Philipp Christen & David E. Metzler, ed. (1985) (John Wiley and Sons, New York). Briefly, a transaminase reaction requires two substrates, an amino acid and a keto acid. The transaminase catalyzes the exchange of the keto group (C=O) from the keto acid and  
20 the amino group from the amino acid (-NH<sub>2</sub>). This exchange generates a new amino acid from the keto acid

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and a new keto acid from the amino acid. Typically only one of the products is desired, generally the new amino acid, and the other is an unwanted by-product.

Used in isolation, the enzyme converts the two

5 substrates to the two products. Theoretically, because the reaction is reversible, it proceeds until it reaches equilibrium producing roughly 50% conversion of the substrate to products.

10 U.S. Patent No. 4,518,692 ("Rozzell I") discloses a method for producing L-amino acids by reacting L-aspartic acid and various 2-keto acids with transaminases. The Rozzell I method uses L-aspartic acid as the amino acid to produce oxaloacetate and

15 describes various methods of decarboxylating oxaloacetate to form pyruvate. However, as will be shown herein, the pyruvate produced in the Rozzell I method can still act as a keto donor in the reverse process to form alanine. Tokarski et al.,

20 Biotechnology Letters, Vol. 10 (1) (1988), pp. 7-10, show that alanine acts as a substrate in transaminase reactions. See also, Transaminases (1985); and Amino Acids: Biosynthesis and Genetic Regulation, Klaus M. Herrmann and Ronald L. Somerville ed. (1983) (Addison-

25 Wesley Publishing, Reading MA). Tokarski, et al.

studied the use of a transaminase to produce L-2-aminobutyrate from 2-ketobutyrate and alanine. The reference, however, discloses only 25-30% conversion to products, demonstrating that the reverse reaction will

30 prevent attaining even the theoretical limit of 50%.

This has long been considered an intrinsic property and a problem of transaminase reactions and is the major reason such enzyme catalyzed reactions have not been exploited more in industrial processes to produce these

35 highly desired amino acid products. The present invention differs from Rozzell I and Tokarski et al. by providing an effective enzymatic method to remove the potential substrates of the reverse reaction from the

mixture.

U.S. Patent No. 4,826,766 ("Rozzell II"), discloses an improved transaminase catalyzed reaction that employs 5 two transaminase enzymes and additional keto acids. In the process, a first transaminase enzyme catalyzes the reaction between a first amino acid and a first keto acid to produce a second amino acid and second keto acid. A second transaminase enzyme then catalyzes a 10 further reaction of the second amino acid and a third keto acid to form the desired amino acid. The two transaminase enzymes are selected such that the first enzyme does not catalyze the second reaction and the second enzyme does not catalyze the first reaction. 15 The Rozzell II method, however, requires additional keto acid and does not disclose the use of acetolactate synthase.

The disclosure of these patents and references are 20 hereby incorporated in their entirety into this specification by reference. Thus, a method to increase the yield of amino acids using transaminase enzymes is desirable.

25 This invention provides an improved transaminase process, which combines the transaminase enzyme with a second enzyme that eliminates the keto acid produced by the transaminase reaction, preventing the formation of equilibrium, and driving the amino acid producing 30 reaction to completion. The second enzyme catalyzes a reaction which converts the keto acid to a substance that can no longer react with the transaminase. By removing the second keto acid, the second enzyme allows 35 the amino acid producing reaction to proceed to an extent that the desired amino acid product represents approximately 100% of the amino acids produced.

The amino acids produced by this process are useful by

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themselves, for example as feed additives, flavor enhancers, sweeteners, and nutritional supplements, or can be used as synthetic intermediates to be further reacted to form useful products, in particular 5 pharmaceuticals. Amino acid products of this process are particularly useful as single enantiomer starting materials for producing chiral pharmaceuticals.

SUMMARY OF THE INVENTION

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This invention provides a process for making an amino acid which comprises reacting a first amino acid, a first keto acid and a transaminase enzyme under conditions appropriate to produce a second amino acid 15 and pyruvate; and reacting the pyruvate with acetolactate synthase under conditions appropriate to produce a compound that does not react with the transaminase enzyme.

20

The invention also provides a process for producing an amino acid which comprises:

- a) reacting a first amino acid with an enzyme under conditions appropriate to produce a keto acid;
- b) reacting the keto acid with a second amino acid 25 and a transaminase enzyme under conditions appropriate to produce a third amino acid and pyruvate; and
- c) reacting pyruvate with acetolactate synthase.

The invention further provides a process for making L- 30 2-aminobutyrate comprising:

- a) reacting L-threonine with threonine deaminase under conditions appropriate to produce 2-ketobutyrate;
- b) reacting the 2-ketobutyrate, L-aspartate and transaminase enzyme under conditions appropriate to 35 produce oxolactate and L-2-aminobutyrate;
- c) allowing the oxolactate to form pyruvate;
- d) reacting the pyruvate with acetolactate synthase enzyme under conditions appropriate to produce

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acetolactate;

- e) allowing the acetolactate to form acetoin; and
- f) separately recovering acetoin and L-2-aminobutyrate.

5

The invention also provides a process for producing 2-amino butyrate which comprises:

a) reacting L-threonine with a threonine deaminase enzyme under conditions appropriate to produce 2-

10 ketobutyrate;

b) reacting 2-ketobutyrate with an amino acid and transaminase enzyme under conditions appropriate to produce 2-aminobutyrate and pyruvate; and

c) reacting pyruvate with acetolactate synthase

15 enzyme under conditions appropriate to produce a compound that does not react with transaminase enzyme.

The invention also provides a reaction medium comprising a keto acid, an amino acid, transaminase 20 enzyme, and acetolactate synthase enzyme.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the production of plasmid pIF349 from 25 plasmid pLG338 discussed in Example 6 below.

Figure 2 shows the production of plasmid pIF306 from plasmid pBR322 discussed in Example 1 below.

30 Figure 3 shows the production of plasmid pIF307 from plasmid pIF306 discussed in Example 1 below.

Figure 4 shows the production of plasmid pIF312 from plasmid pIF307 discussed in Example 1 below.

35

Figure 5 shows the production of plasmid pIF322 from plasmid pIF312 discussed in Example 5 below.

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Figure 6 shows the production of plasmid pIF347 from plasmid pIF312 discussed in Example 2 below.

5 Figure 7 shows the production of plasmid pPT353 from plasmid pIF312 discussed in Example 1 below.

Figure 8 shows plasmid pIF328 discussed in Example 7 below.

10 Figure 9 shows plasmid pME64 discussed in Example 3 below.

Figure 10 shows plasmid pPOT1 discussed in Example 1 below.

15 Figure 11 shows the production of plasmid pPOT2 from plasmid pPOT1 discussed in Example 1 below.

20 Figure 12 shows the production of plasmid pPOT3 from plasmid pPOT2 discussed in Example 1 below.

Figure 13 shows the production of plasmid pPOT300 from plasmid pPOT3 discussed in Example 4 below.

25 Figure 14 shows the production of plasmid pPPT368 from plasmid pPOT3 discussed in Example 8 below.

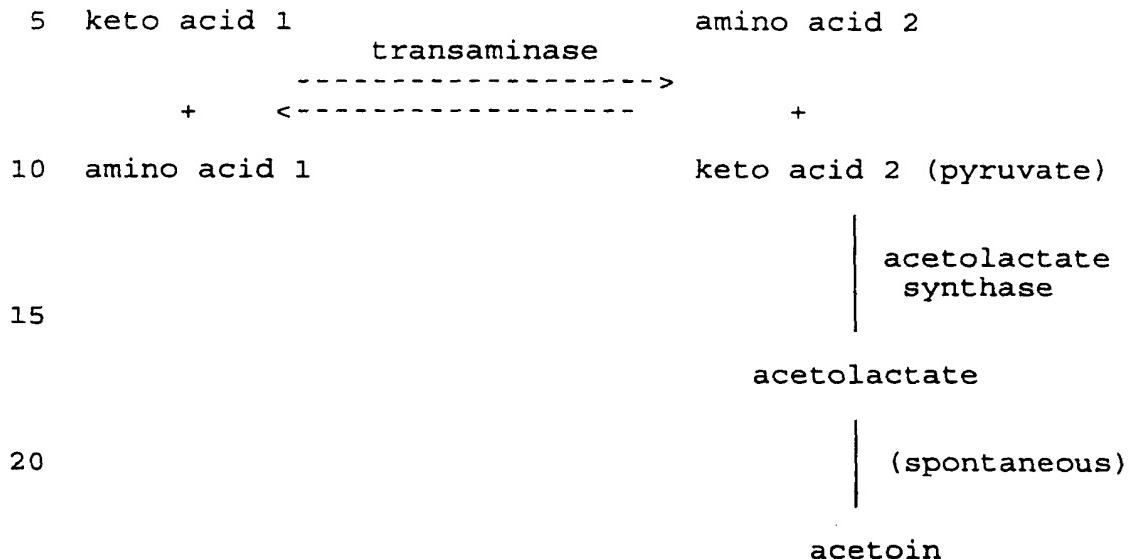
#### DETAILED DESCRIPTION OF THE INVENTION

30 This invention provides a process for making an amino acid which comprises reacting a first amino acid, a keto acid and a transaminase enzyme under conditions appropriate to produce a second amino acid and pyruvate; and reacting the pyruvate with acetolactate synthase under conditions appropriate to produce a compound that does not react with the transaminase enzyme.

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The process of this invention is schematically described as follows:

Scheme 1



- 25 In the practice of this invention the first amino acid, sometimes referred to herein as the "amino donor", includes any amino acid that will react with a transaminase and a keto acid, sometimes referred to herein as the "keto donor" to produce the desired amino acid product ("amino acid 2" in Scheme 1) and pyruvate ("keto acid 2" in Scheme 1). In one embodiment the first amino acid is alanine, which directly forms pyruvate under such conditions. See, Tokarski et al. In a preferred embodiment the first amino acid is L-aspartate. The transaminase reaction of a keto acid with L-aspartate produces oxaloacetate as the second keto acid. Contrary to the disclosure of Rozzell I, those of ordinary skill in the art recognize that under normal reaction conditions oxaloacetate undergoes a spontaneous loss of CO<sub>2</sub> to form pyruvate.

The process of this invention removes the pyruvate by the reaction of the second enzyme, acetolactate synthase, which condenses two molecules of the pyruvate 45 to produce acetolactate. During the condensation the

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carboxyl group leaves as a molecule of carbon dioxide, rendering this reaction essentially irreversible. Acetolactate then undergoes a further spontaneous decarboxylation to produce acetoin, which is volatile  
5 and easy to separate from the amino acid product of the reaction using known techniques to afford a purified amino acid product if desired.

Transaminases with varying selectivities are known.  
10 See Transaminases, (1985); Amino Acids: Biosynthesis and Genetic Regulation (1983); and U.S. Patent No. 4,826,766 (Rozzell II). The processes of the invention are intended to encompass the use of any natural or non-naturally occurring microorganism, such as a  
15 bacterium or virus, that produces transaminase enzymes. As used herein, the term "non-naturally occurring microorganism" is intended to include all genetically altered microorganisms that produce transaminase enzymes. For example, Rozzell II discloses several  
20 microorganism sources for transaminases having selectivities for aromatic amino acids, branched chain amino acids, and amino acids having acidic side chains.

In one embodiment of the process, the transaminase  
25 enzyme is produced by cells of a non-naturally occurring microorganism that contains a gene encoding a transaminase enzyme. For example, the gene encoding the transaminase can be incorporated into a plasmid that is inserted into a cell such that the cell  
30 produces the transaminase enzyme. In another embodiment of the invention a multiplicity of transaminase enzymes, for example 2, 3, 4, and the like, can be simultaneously utilized in the process. Thus, as used herein "transaminase enzyme" can comprise  
35 one or more than one transaminase enzymes being used simultaneously. In a preferred embodiment, whether is a single transaminase enzyme or as one of a multiplicity of transaminase enzymes, the transaminase

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enzymes used is the enzyme produced by plasmid pME64 described herein.

A general description of sources of acetolactate synthases can be found in Renna, M.C. et al., J. Bacteriol., (1993) Vol. 175, pp. 3863-3875; and Wek, R.C. et al., Nucleic Acids Res., (1985) Vol. 13, pp. 3995-4010. These references are hereby incorporated in their entirety into this specification by reference. As noted above for the transaminase enzyme, the processes of the invention are intended to encompass the use of any natural or non-naturally occurring microorganism, such as a bacterium or virus, that produces acetolactate synthase enzymes. In one embodiment of the process, the acetolactate synthase enzyme is produced by cells of a non-naturally occurring microorganism that contains a gene encoding a transaminase enzyme. For example, the gene encoding the acetolactate synthase can be incorporated into a plasmid that is inserted into a cell such that the cell produces the acetolactate synthase enzyme. In another embodiment of the invention a multiplicity of acetolactate synthase enzymes, for example 2, 3, 4, and the like, can be simultaneously utilized in the process. Thus, as used herein "acetolactate synthase enzyme" can comprise one or more than one acetolactate synthase enzymes being used simultaneously. In a preferred embodiment, whether as a single acetolactate synthase enzyme or as one of a multiplicity of acetolactate synthase enzymes, the acetolactate synthase enzyme is the enzyme produced by plasmid pPOT300 described herein.

The process of this invention can be applied to produce a variety of natural and non-naturally occurring amino acids simply by selecting the appropriate keto acid. U.S. Patent No. 4,518,692 and Transaminases, (1985) for disclosures of a broad range of keto acids which are

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useful in this invention. In a preferred embodiment, the keto acid is 2-ketobutyrate. In a separately preferred embodiment, the keto acid is tri-methyl pyruvate.

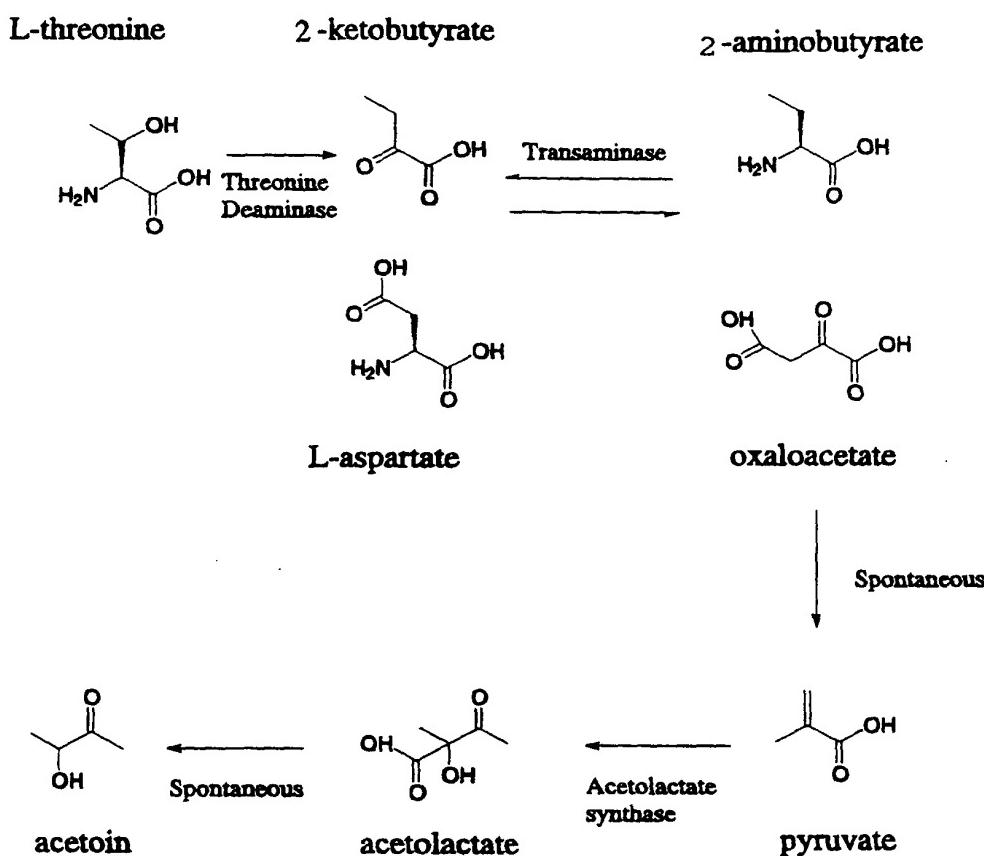
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In addition to these sources, keto donors can also be prepared from readily available starting materials, including other amino acids. For example, the enzyme threonine deaminase reacts with L-threonine to produce

- 10 2-ketobutyrate. See Scheme 2 below. The keto acid, thus produced, is then reacted with the L-aspartate amino acid substrate according to the process described above to produce L-2-aminobutyrate. L-threonine is a inexpensive starting material available from Archer  
15 Daniels Midland (Decatur, IL) and the reaction produces 2-ketobutyrate in essentially 100% yield. See, Amino Acids: Biosynthesis and Genetic Regulation (1983)

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Scheme 2



Additional reactions using various amino acid starting materials to produce various keto acids that are useful in this process are known in the art. Gene, (1989) Vol. 76, pp. 255-269 and Gene, (1988) Vol. 63, pp. 245-252 describe. See also, Massad G., et al., J.Bacteriol., (1992) Vol 177, pp. 5878-5883, for a general description of the activity of amino acid deaminase

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enzymes from *Proteus mirabilis*. These references are hereby incorporated in their entireties into this specification.

5 Thus, this invention also provides a process for producing an amino acid which comprises:

a) reacting a first amino acid with an enzyme under conditions appropriate to produce a keto acid;

10 b) reacting the keto acid with a second amino acid and a transaminase enzyme under conditions appropriate to produce a third amino acid and pyruvate; and

c) reacting pyruvate with acetolactate synthase.

Reaction of the pyruvate in step (c) proceeds according  
15 to the description above to produce acetoin which can be readily separated from the third amino acid to isolate the amino acid product. In a preferred embodiment the enzyme in step (a) is a deaminase enzyme. In a particularly preferred embodiment the  
20 first amino acid is threonine and the enzyme is threonine deaminase.

In a preferred embodiment, the invention provides a process for producing 2-amino butyrate which comprises:

25 a) reacting L-threonine with a threonine deaminase enzyme under conditions appropriate to produce 2-ketobutyrate;

b) reacting 2-ketobutyrate with an amino acid and a transaminase enzyme under conditions appropriate to  
30 produce 2-aminobutyrate and pyruvate; and

c) reacting pyruvate with acetolactate synthase.

In the practice of these methods the enzyme used in step (a), such as a deaminase, can be from any of the  
35 sources described above, or can be a multiplicity of enzymes as described above. In one embodiment of the invention a multiplicity of enzymes, for example 2, 3, 4, and the like, can be simultaneously utilized in the

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process. Thus, as used herein "deaminase enzyme" can comprise one or more than one deaminase enzymes being used simultaneously. In a preferred embodiment of this process the deaminase enzyme is a threonine deaminase 5 enzyme produced by plasmid pIF347 described herein. In the practice of this method of the invention the amino acid and the transaminase enzyme used in step (b) can be any amino acid and transaminase enzyme as described above that form pyruvate. In a preferred embodiment 10 the amino acid in step (b) is L-aspartate. In a separately preferred embodiment the transaminase enzyme is an enzyme produced by the plasmid pME64. The acetolactate synthase enzyme used in step (c) can be from any of the sources described above. In a 15 preferred embodiment of the invention, the acetolactate synthase enzyme is the enzyme produced by the plasmid pPOT300.

Techniques for Utilizing Enzymes

- 20 In the practice of this invention "conditions appropriate" to react the described enzymes with the described substrates are known to those of ordinary skill in the art.
- 25 For example, cells producing transaminase and acetolactate synthase enzymes may be contacted with a solution containing the keto acid and amino acid starting materials with the resulting conversion of at least a portion of the keto acid starting material in 30 the reaction mixture to the desired amino acid product. The cells may be permeabilized to facilitate diffusion of the substrates and products into and out of the cells. This permeabilization can be accomplished by treating cells with a low concentration of a 35 surfactant, including but not limited to Tween 80®, Triton X-100, Nonidet P40®, cetylpyridinium chloride, deoxycholic acid, hexadecyltrimethylammonium bromide, benzalkonium chloride. Further, organic solvents,

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including but not limited to N,N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), ethanol or acetone at low concentrations have also been used to increase permeabilization. Enzymes, including the transaminase, 5 threonine deaminase, and the acetolactate synthase, may also be added to the starting reaction mixture in the form of cell extracts containing crude, partially purified, or purified enzyme. Cell extracts are prepared by methods known to those skilled in the art 10 which provide for cell disruption and recovery of the enzyme. Cell disruption, can be accomplished by mechanical or non-mechanical means. Most often, for bacterial suspensions mechanical devices such as a French pressure cell, ultrasonication, bead mill or 15 Manton-Gaulin homogenizer is used with the specifics of the method known to those of ordinary skill in the art. See, Scopes, R.K. "Protein Purification", (1982) (Springer-Verlag, New York). The reaction using the cell extract is then carried out in similar fashion to 20 the whole cell method discussed above.

The enzyme-containing cells, or extracts thereof or purified enzyme or enzyme fractions, may also be immobilized, if desired. Immobilization methods which 25 may be used in the practice of this invention include well-known methods such as entrapment in polymeric gels, covalent attachment, crosslinking, adsorption, and encapsulation. Some examples of these methods are described by A.M. Klibanov in Science, 219:722-727 30 (1983) and the references therein and in Methods in Enzymology (1976), Volume 44, (K. Mosbach editor) which are hereby incorporated by reference. In one method of immobilization disclosed in U.S. Patent No. 5,019,509, a support material containing at least 20% by weight of 35 silica or alumina is contacted with aminoalkyl compound such as an aminoalkyl silane, polyethyleneimine, or a polyalkylamine, followed by activation with glutaraldehyde. The enzyme-containing solution is then contacted

- 15 -

with the activated support to produce an immobilized enzyme composition having transaminase- and/or acetolactate synthase activity. Other immobilization supports useful in the practice of this invention 5 include, but are not limited to, porous glass and porous ceramics, bentonite, diatomaceous earth, charcoal Sepharose® and Sepharose® derivatives, cellulose and cellulose derivatives, polyacrylamide and polyacrylamide derivatives, polyazetidine, alginate, 10 carrageenan, and Chromosorb®. Sepharose® (Pharmacia Fine Chemicals, Uppsala Sweden) is a bead-formed gel prepared from agarose. The manufacturer's product literature reports that in its natural state, agarose occurs as part of the complex mixture of charged and 15 neutral polysaccharides referred to as agar. The agarose used to make Sepharose® is obtained by a purification process which removes the charged polysaccharides to give a gel with only a very small number of residual charged groups. Those of ordinary 20 skill in the art will appreciate that a number of other materials suitable for the immobilization of cells or extracts derived therefrom may also be useful for the immobilization of the enzymes used in the present invention. These supports can be activated, if 25 desired, by techniques well-known in the art.

The reaction to produce a desired amino acid product utilizing cells containing transaminase and acetolactate synthase, or compositions comprising 30 extracts derived from said cells, is carried out by contacting a solution containing a first keto acid and a first amino acid with the enzymes under conditions permitting the conversion of at least a portion of the first keto acid to the desired amino acid. In the 35 practice of the processes of this invention the cells contact an aqueous solution of the enzymes at a cell load in the range of about 50 mg/ml to about 200 mg/ml. In a preferred embodiment the cell load is about 100

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mg/ml. When the invention is practiced using extracts of cells, the extracts are prepared from an amount of cells that would give these cell loads.

- 5 The enzymatic reactions of this invention are carried out at temperatures in the range of from about 30°C to about 50°C, and preferably at temperatures ranging from about 37°C to about 45°C. The optimal pH for the reaction ranges from about 6 to about 9, and more 10 preferably from about 7 to about 8, with a pH of 8 being most preferred.

15 The invention will now be further illustrated by the following examples, which are not intended, and should not be interpreted, to limit the scope of the invention which is defined in the claims which follow thereafter.

Experimental Details

- 20 Examples 1-4 show the production of plasmids containing genes which encode for the preferred enzymes used in a preferred embodiment of the process of the claimed invention, the production of L-2-aminobutyrate. In the Examples these plasmids, pIF347, pME64, and pPOT300, 25 were used separately in individual bacterial host strains, each utilizing host strain W3110. W3110 is obtained from the ATCC (Rockville, MD) (ATCC Accession No. 27325). However, those of ordinary skill in the art will recognize that other individual host strains 30 will be appropriate in the practice of this invention. In addition, those of ordinary skill will recognize that more than one or all plasmids can be incorporated into a single host strain and utilized as described above.

35

General molecular biology techniques known to those of ordinary skill in the art are used throughout. See, for example, Molecular Cloning, Sambrook et al. eds.

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(Second ed. 1989) (Cold Spring Harbor Publications, NY). PCR methods were carried out using a Perkin Elmer GeneAmp kit supplied by Roche (Branchburg, NJ) according to the instructions supplied. Ligations were 5 performed using a kit supplied by Takara Biochemical (Pan Vera Corp., Madison, WI) according to the instructions supplied. DNA cleavages were performed using restriction enzymes supplied by New England Biolabs (Beverly, MA) according to the instructions 10 supplied. Chromosomal DNA was prepared using Qiagen genomic DNA kits (Qiagen, Santa Clarita, CA) according to the instructions supplied. Qiagen further supplied PCR purification kits, which were used according to the instructions supplied.

15

The expression of the genes was carried out on a series of expression vectors that represent a preferred embodiment of the invention, and which are referred to herein as pPOT. These plasmids enable one to turn on 20 the production of the enzymes at a particular stage in the growth of the cells by adjusting the temperature of the fermentor. The plasmids carry a mutated version of a repressor named cI from phage lambda. See, Lambda II, R.W. Hendrix et al., eds. (1983) (Cold Spring 25 Harbor Publications, NY); and Stratagene Cloning Systems Product Catalog (Stratagene Cloning Systems, La Jolla, CA). The mutation makes the repressor unstable at elevated temperatures such as those above about 30°C. The repressor normally prevents expression of 30 genes under control of the promoter  $P_R$ . When the temperature is raised the repressor becomes inactive and the promoter begins to work. In each case the gene of interest is placed under control of the promoter  $P_R$ .

35

Example 1

Construction of the Expression Vectors  
pPOT1, pPOT2 and pPOT3

Plasmid pPOT1. Plasmid pBR322 was obtained from New

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England Biolabs (Beverly, MA). A modified *pheA* promoter was inserted between unique HindIII and SphI sites on pBR322 to construct pIF306. Within the HindIII to SphI insert there exists unique BamHI and 5 BglII sites. The modified *pheA* promoter was derived from that characterized in co-owned U.S. Patent No. 5,120,837 to Fotheringham et al. which is incorporated by reference herein, such that the sequence was as follows:

10

HindIII

AAGCITTTTGTGACAGCGTGAAAACAGTACGGGTATAATACT  
AAAGTCACAAGGAGGATCCACTATGACATCGGAAAACCCGTTACT  
GGCGCT

15

HaeII

20 pIF306 was cleaved with the enzymes BamHI and SphI. The 3.9 kB fragment was isolated and ligated to a similarly cleaved fragment containing the *E. coli* K12 *ilvE* gene which was generated by PCR from W3110 chromosome using the following oligonucleotide primers:

5' CGC GGA TCC ACT ATG ACC ACG AAG AAA GCT GAT TAC ATT TGG 3'

25

5' CAG CGT GCA TGC TTA TTG ATT AAC TTG ATC TAA CCA GC 3'

30

The resulting vector was named pIF307. Plasmid pIF307 was cleaved with enzymes EcoRI and PstI and the 4.1 kB fragment isolated. This was ligated to a similarly cleaved and purified 982 base pair DNA fragment containing the kanamycin resistance gene from plasmid pLG338 (pLG338 is described in US patent 5,120,837 and Stoker et al Gene 18:, 335-341). This was generated using PCR with the following oligonucleotide primers:

35

5' CCG GAA TTC ACG TTG TGT CTC AAA ATC TCT GAT 3'

5' CCG CTG CAG GCC GTC CCG TCA AGT CAG CGT AAT G 3'

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The resulting plasmid was named pIF312. Plasmid pIF312 was cleaved by EcoRI and BamHI and the resulting 4.97 kB fragment was ligated to the phage lambda *cI857* gene which was similarly cleaved following isolation by PCR 5 using the Lambda ZapII vector (Stratagene, La Jolla, CA) as template and the following oligonucleotide primers:

5' TTTGGATCCTCCTTAGTACATGCAACC 3'

10

5' TTTGAATTCGGATGAAGATTCTTGCTCGATTGT 3'

The resulting plasmid was named pPT353. This plasmid was then cleaved with PstI and EagI and the 3.17kB 15 fragment was isolated. Plasmid pPOT1 can then be constructed by ligating this fragment to the 1.95kB PstI to BspEI fragment of pBR322 and the PCR fragment generated from pLG338 using the oligonucleotides:

20 5' GGC GGC CGA CGC GCT GGG CTA CG 3'

5' CCC TCG CAA GCT CGT CCG GAG GCA AAT CGC TGA ATA TTC C 3'

This PCR results in the amplification of a 0.59kB DNA 25 fragment. This fragment is then cleaved with BspEI and EagI to generate the necessary sticky ends for the trimolecular ligation to generate pPOT1.

To generate plasmid pPOT2, pPOT1 was then cleaved with 30 BamHI and Sall and the 4.68 kB fragment isolated. This fragment was ligated to an oligonucleotide linker prepared by annealing the following two oligonucleotides:

35 5' GATCCTAGGTACCGGTGCGGCCGCATGCTGACTGACTGAAGATCCGGCGATTCT  
ACGCCCGGGTTTTATG 3'

5' TCGACATAAAAACCCGGCGTAGAACATGCCGGATCTTCAGTCAGTCAGCATGC

- 20 -

GGCCGCACCGGTACCTAG 3'

The resulting plasmid was named pPOT2. This plasmid was cleaved with XhoI and PstI and the 3.9 kb fragment 5 isolated. This was ligated to a fragment similarly cleaved with XhoI and PstI containing the cat gene of HSG415 described in U.S. Patent No. 5,345,672 (Fotheringham), the disclosure of which is hereby incorporated into this specification by reference. The 10 cat gene confers chloramphenicol resistance upon the host strain and can be isolated from plasmid HSG415 using the following oligonucleotide primers:

5' GAC CTC GAG GCA CTT TGC GCC GAA TAA ATA CCT GTG 3'

15

5' GAC CTG CAG CAC CAG GCG TTT AAG GGC ACC AAT AAC 3'

The resulting plasmid was named pPOT3.

20

#### Example 2

##### Plasmid pIF347 Comprising the *E. coli* *ilvA* Gene Encoding Threonine Deaminase

To construct pIF347 the *ilvA* gene encoding threonine 25 deaminase was amplified from the chromosome of *E. coli* K12 by PCR. *E. coli* K12 chromosomal DNA was prepared using standard methodology. The *ilvA* gene was specifically amplified by PCR using the oligonucleotide primers:

30

5' CGC GGA TCC ATC ATG GCT GAC TCG CAA CCC C 3'

5' CTC GCA TGC CAG GCA TTT TTC CCT AAC CCG CC 3'

35 The PCR product was cleaved using the restriction enzymes BamHI and SphI and the 1.57kB fragment thus generated was ligated to the 4.1kB fragment of pIF312 which had been similarly cleaved with BamHI and SphI.

- 21 -

The resulting plasmid was named pIF347.

Example 3

5 Plasmid pME64 Comprising the *E. coli* *tyrB* Gene Encoding Tyrosine Aminotransferase

Plasmid pME64, which contains a fragment of the *E. coli* K12 chromosome encoding the *tyrB* gene, is described in Fotheringham et al., *Biochemical Journal*, (1986) Vol. 10 234, pp. 593-604, the contents of which are hereby incorporated into this specification by reference. The *tyrB* gene may also be isolated directly from the chromosome of *E. coli* K12 using oligonucleotide primers based on the sequence disclosed in the reference using 15 standard techniques.

Example 4

20 Plasmid pPOT300 Comprising the *B. subtilis* *alsS* Gene Encoding Acetolactate Synthase

The *alsS* gene was amplified from the *B. subtilis* chromosome using PCR. *B. subtilis* chromosomal DNA was prepared using the same procedure as for *E. coli* K12. The *alsS* gene was specifically amplified by PCR using 25 the following oligonucleotide primers.

5' TTT GAA TCC ATC ACA AGA TAT TTA AAA TTT 3'

5' TTT AGC GTC GAC GCA TGC TCC TTT TAT TTA GTG CTG TTC 3'

30 The PCR product was then cleaved with the enzymes BamHI and SalI and the resulting 1.9 kB fragment was ligated to the 4.76kB fragment of pPOT3 which was isolated by agarose gel electrophoresis following similar cleavage 35 by BamHI and SalI. The resulting plasmid was named pPOT300.

Example 5

Construction of Plasmid pIF322

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The *aspC* gene is also described in the publication Fotheringham et al., (1986). Based upon the sequence disclosed in this citation, the coding sequence of the gene can be isolated from the chromosome of *E.coli* K12 5 as a 1.2 kb fragment by PCR using the following oligonucleotide primers:

5' CGC GGA TCC ACT ATG TTT GAG AAC ATT ACC GCC 3'

10 5' CAG CGT GCA TGC TTA CAG CAC TGC CAC AAT CGC 3'

To construct pIF322, plasmid pIF312 was cleaved with BamHI and SphI and the 4.1kb fragment isolated as before. This was ligated to the *aspC* containing PCR 15 fragment to give pIF322.

Example 6

Construction of Plasmid pIF349

20 Based upon the sequence disclosed in the above citation, the *aspC* gene along with the wild type promoter region can be isolated using as a 1.6 kb fragment by using the following oligonucleotide primers:

25 5' GAC GGA TTC CCA GAG CAA TCT CAC GTC TTG C 3'  
5' GAC AGA TCT CCC TGA TAA GCG TAG CGC ATC AGG 3'

30 Following purification of the PCR product using a Qiagen PCR purification kit and digestion with EcoRI and BglII, the fragment can be ligated to the 7.1kb EcoRI to BamHI fragment of plasmid pLG338. Plasmid pLG338 is described in U.S. Patent No. 5,120,837 and 35 and in Stoker et al., Gene Vol. 18, pp. 335-341, the contents of which are hereby incorporated into this specification by reference. The resulting plasmid is pIF349.

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Example 7

Construction of Plasmid pIF328

Plasmid pIF328 is derived from pIF312. Initially the  
5 pheA derived promoter region of pIF312 was replaced by  
one which was derived from the *pckA* promoter of *E.coli*  
K12. This is described in the GENBANK entry ECOPCKA.  
The *pckA* promoter was amplified on a 270 bp fragment  
using the following oligonucleotide primers:

10

5' GAC GAA TTC ACT TTA CCG GTT GAA TTT GC 3'

5' GAC GGA TCC TCC TTA GCC AAT ATG TAT TGC 3'

15 15 A mutation was introduced in the -10 region of the *pckA*  
promoter using standard PCR based mutagenesis  
procedures such that the wild type -10 sequence of 5'  
GATAAT 3' was changed to 5' TATAAT 3'. This resulted  
in the elimination of the normal catabolite repression  
20 of the promoter.

The fragment was isolated and purified using a Qiagen  
PCR purification kit and ligated to the 4.97kb fragment  
of pIF312 generated by cleavage with EcoRI and BamHI.

25 25 The resulting plasmid was pIF313.

Plasmid pIF313 was then cleaved with the enzymes BspEI  
and SphI and the 4.14kb fragment isolated and purified  
using a Qiagen PCR purification kit. The Par,  
30 partition locus of pLG338 was then amplified by PCR  
using the following oligonucleotide primers:

5' CTT GCA TGC ACC ATT CCT TGC GGC GGC 3'

35 35 5' AGA TCC GGA GGC AAA TCG CTG AAT ATT CC 3'

The resulting 970bp fragment was cleaved with BspEI and  
SphI and ligated to the pIF313 fragment. The resulting

- 24 -

plasmid was pIF328.

Example 8

Construction of Plasmid pPT368

5

To construct pPT368, plasmid pPOT3 was cleaved by the enzymes BamHI and SphI and the 4.8kb fragment isolated. The E.coli K12 *ilvE* gene was amplified from the E.coli chromosome by PCR using the following oligonucleotide primers:

5' CGC GGA TCC ACT ATG ACC ACG AAG AAA GCT GAT  
TAC ATT TGG 3'

15 5' CAG CGT GCA TGC TTA TTG ATT AAC TTG ATC TAA  
CCA GC 3'

This 0.94 kb fragment was purified using a Qiagen PCR purification kit, similarly cleaved with BamHI and SphI 20 and ligated to the fragment of pPOT3. The resulting plasmid was pPT368.

In the following Examples 9 and 10 the effect of acetolactate synthase upon the efficiency of 25 2-aminobutyrate biosynthesis was investigated using 2-ketobutyrate or L-threonine as substrate. Cell cultures of W3110 cells carrying pME64, pPOT300 or pIF347 were prepared by inoculating 50mls of LB medium with a single colony from an LB agar plate and 30 culturing overnight at 37°C in an 500ml flask in a shaking incubator. Antibiotics, where appropriate, were added at the concentrations of 100µg/ml ampicillin, 40µg/ml kanamycin and 10µg/ml chloramphenicol. The plasmid descriptions from the 35 Examples above show which antibiotic is required by each plasmid. Overnight cultures were then used to inoculate 1 liter of LB plus appropriate antibiotics to an initial OD<sub>600</sub> of 0.05. These were grown at 37°C in

- 25 -

a 4 litre flask with agitation at 300rpm until the OD600 reached 1.0. The cells were then recovered by centrifugation at 10,000G for 5 minutes washed in 50mM Tris HCl buffer and similarly pelleted. The required weight of pelleted wet cells was then added to bioconversion mixes.

HPLC analysis for amino acids were carried out as follows.

10

1. L-2-aminobutyrate:

Methods: OPA/BOC-Cys derivatization.

Mobile Phase: Gradient method, Pump A = 60% MeOH, 40% 0.05M TEAP, buffer pH = 7.0; Pump B = H<sub>2</sub>O; Pump B 32% at 0 min. and at 6 min., 5% by 8 min. At 14.1 min. revert to starting conditions. Oven 40°C.

Column: Supelcosil LC-18DB, 3μ, 150 x 4.6mm

20

Flow rate: 1.0 ml/min

Detection: UV @ 338nm

Injection Volume: 10μl

25 2. L-tert-leucine:

Methods: OPA/BOC-Cys derivatization.

Mobile Phase: Stock solution 300mM NaHPO<sub>4</sub>, pH 7.0; 11.635g NaH<sub>2</sub>PO<sub>4</sub> and 30.723g Na<sub>2</sub>HPO<sub>4</sub> dissolved in 1L water; Pump A = 15mM NaHPO<sub>4</sub>; 50 ml stock diluted to 1L with water; Pump B = 900ml of Pump A solution and 1100ml of ACN.

Column: Supelcosil LC-18DB, 3μ, 150 x 4.6mm

Flow rate: 1.5 ml/min

Detection: UV @ 338nm

35

Injection Volume: 10μl

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Example 9

Biosynthesis of 2-aminobutyrate  
Using 2-ketobutyrate as Substrate.

5 The reaction was run using the following substrate concentrations:

Reaction A

Reaction volume 2ml

10 2-ketobutyrate 500mM (Adjusted to pH 7.5 with NaOH)

L-aspartic acid 500mM (Adjusted to pH 7.5 with NaOH)

100mM Tris pH 7.5

Reaction time 24hrs

100mg/ml Cells of W3110 containing pME64

15

Reaction B

Reaction volume 2ml

2-ketobutyrate 500mM (Adjusted to pH 7.5 with NaOH)

L-aspartic acid 500mM (Adjusted to pH 7.5 with NaOH)

20 100mM Tris pH 7.5

Reaction time 24hrs

100mg/ml cells of W3110 containing pME64 and 50mg/ml cells of W3110 containing pPOT300

25 Following incubation for 24hrs a 200 $\mu$ l sample was taken from each reaction and the cells removed by centrifugation. The sample was then diluted 100 fold and subjected to amino acid analysis by HPLC (details to follow).

30

In the following Tables "L-2-aba" refers to L-2-amino butyrate, "L-ala" refers to content of L-alanine, "L-asp" refers to L-aspartic acid and "L-thr" refers to L-threonine, "L-t-leu" refers to content of L-tert-

35 leucine, "L-glu" refers to content of L-glutamic acid. All content values in the Tables are in mg/ml.

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Table 1

Reaction	L-2-aba	L-ala	L-asp
A	23.89	9.62	1.16
B	30.04	1.65	1.28

5

Example 10Biosynthesis of 2-aminobutyrate  
Using L-threonine as Substrate

- 10 The reaction was run using the following substrate concentrations:

Reaction A

Reaction volume 2ml

- 15 L-threonine 500mM (Adjusted to pH 8.0 with NaOH)  
 L-aspartic acid 500mM (Adjusted to pH 8.0 with NaOH)  
 Reaction time 20hrs  
 100mg/ml cells of W3110 containing pME64  
 50 mg/ml cells of W3110 containing pIF347

20

Reaction B

Reaction volume 2ml

L-threonine 500mM (Adjusted to pH 8.0 with NaOH)  
 L-aspartic acid 500mM (Adjusted to pH 8.0 with NaOH)

- 25 Reaction time 20hrs

100mg/ml cells of W3110 containing pME64  
 50 mg/ml cells of W3110 containing pIF347 and 50mg/ml  
 cells of W3110 containing pPOT300

- 30 Following incubation for 24hrs a 200 $\mu$ l sample was taken from each reaction and the cells removed by centrifugation. The sample was then diluted 100 fold and subjected to amino acid analysis by HPLC.

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Table 2

Reaction	L-2-aba	L-ala	L-asp	L-thr
A	25.05	10.60	0.69	und.
B	27.71	1.23	1.01	und.

5

"Und" represents not detected.

The results show that the use of acetolactate synthase results in very significantly less accumulation of  
 10 L-alanine in the biotransformation. This is true for biotransformations using either 2-ketobutyrate or L-threonine as the keto acid source.

In the case of 2-ketobutyrate the yield and relative  
 15 purity are:

Table 3

	L-2-aba yield from substrate	L-2-aba/L-alanine ratio
- alsS	46.4%	2.4:1
+ alsS	58.3%	18.2:1

In the case of L-threonine the yield and relative purity are:

25

Table 4

	L-2-aba yield from substrate	L-2-aba/L-alanine ratio
- alsS	46.4%	2.4:1
+ alsS	53.8%	22.5:1

30

In each case the yield of product is improved to a moderate degree and the 2-aba:alanine ratio is dramatically improved roughly sevenfold and tenfold.

35 Although in these examples the genes were each present on a different plasmid in independent strains, it would be a logical step to combine the genes on one or more plasmids in a single strain. This would reduce the time, complexity and cost of fermenting the organisms

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for large scale manufacture of L-2-aba or other amino acids using this approach. In addition, those of ordinary skill in the art will recognize that separate genes encoding for more than one transaminase, 5 deaminase and/or acetolactate synthase enzyme can be incorporated onto a single plasmid.

Example 10  
Comparative Example

10 Biosyntheses of L-tert-leucine Using  
Coupled transaminases With and Without alsS

The following reaction provides a comparison of the claimed methods with process disclosed in Rozzell I 15 patent discussed above.

Reaction A

Reaction volume 1 litre

Tri-methyl pyruvate 500mM

20 L-aspartic acid 550mM

L-glutamic acid 50mM

pH 8.0

Reaction time 48hrs

100mg/ml cells of W3110 containing pIF349 and pPT368

25

Reaction B

Reaction volume 1 litre

Tri-methyl pyruvate 500mM

L-apartic acid 550mM

30 L-glutamic acid 50mM

pH 8.0

Reaction time 48hrs

100mg/ml Cells of W3110 containing pIF328

50mg/ml Cells of W3110 containing pIF322

35 50mg/ml Cells of W3110 containing pPOT300

Following incubation for 48 hours a 1 ml sample was taken from each reaction and the cells removed by centrifugation. The sample was then diluted 100 fold

- 30 -

and subjected to amino acid analysis by HPLC.

Table 5

Reaction	L-t-leu	L-ala	L-asp	L-glu
A	43.9	11.6	0.0	5.7
B	48.1	6.0	1.5	5.6

These results show that the reaction as described in the Rozzell I, although showing complete conversion of the amino donor to product, does lead to the accumulation of alanine as a contaminating amino acid. Also, the addition of acetolactate synthase enzyme in the reaction leads to a reduction of alanine biosynthesis. The result can be expressed as a ratio of L-tert-leucine to L-alanine. In Reaction A (-alsS) the ratio is 3.78. In Reaction B (+alsS) the ratio is 8.02.

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What is claimed is:

1. A process for making an amino acid which comprises reacting a first amino acid, and a keto acid with transaminase enzyme under conditions appropriate to produce a second amino acid and pyruvate; and reacting the pyruvate with acetolactate synthase enzyme under conditions appropriate to produce a compound that does not react with transaminase enzyme.
- 10 2. The process of Claim 1 wherein the compound that does not react with transaminase enzyme is acetolactate.
- 15 3. The process of Claim 1 wherein the compound that does not react with transaminase enzyme is acetoin.
4. The process of Claim 1 wherein the first amino acid is alanine.
- 20 5. The process of Claim 1 wherein the first amino acid is L-aspartate.
6. The process of claim 1 wherein the keto acid is 2-ketobutyrate.
- 25 7. The process of claim 1 wherein the keto acid is tri-methyl pyruvate.
8. The process of Claim 1 wherein transaminase enzyme is present in aqueous solution as whole cells producing transaminase enzyme.
- 30 35 9. The process of Claim 8 wherein the whole cells are present in aqueous solution at a cell load of about 50 mg/ml to about 200 mg/ml.
10. The process of Claim 9 wherein the whole cells are present in aqueous solution at a cell load of about 100

mg/ml.

11. The process of Claim 1 wherein transaminase enzyme  
is present in aqueous solution as cell extracts of  
5 whole cells producing transaminase enzyme.

12. The process of Claim 1 wherein transaminase enzyme  
is present as immobilized enzyme.

10 13. The process of Claim 1 wherein transaminase enzyme  
is produced by a cell from a non-naturally occurring  
microorganism comprising a gene that encodes for  
transaminase enzyme.

15 14. The process of Claim 13 wherein the cell comprises  
a plasmid that comprises a gene that encodes for  
transaminase enzyme.

15. The process of Claim 14 wherein the plasmid is  
20 pME64.

16. The process of Claim 1 wherein acetolactate  
synthase enzyme is present in aqueous solution as whole  
cells producing acetolactate synthase enzyme.

25 17. The process of Claim 16 wherein the whole cells  
are present in aqueous solution at a cell load of about  
50 mg/ml to about 200 mg/ml.

30 18. The process of Claim 17 wherein the whole cells  
are present in aqueous solution at a cell load of about  
100 mg/ml.

19. The process of Claim 1 wherein acetolactate  
35 synthase enzyme is present in aqueous solution as cell  
extracts of whole cells producing acetolactate synthase  
enzyme.

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20. The process of Claim 1 wherein acetolactate synthase enzyme is present as immobilized enzyme.

21. The process of Claim 1 wherein acetolactate synthase enzyme is produced by a cell from a non-naturally occurring microorganism comprising a gene that encodes for acetolactate synthase enzyme.

22. The process of Claim 21 wherein the cell comprises a plasmid that comprises a gene that encodes for acetolactate synthase enzyme.

23. The process of Claim 22 wherein the plasmid is pPOT300.

15

24. The process of Claim 1 further comprising the step of isolating the second amino acid.

25. A process for making an amino acid comprising:

20 a) reacting a first amino acid with an enzyme under conditions appropriate to produce a keto acid;

b) reacting the keto acid from (a) with a second amino acid and a transaminase enzyme under conditions appropriate to produce a third amino acid and pyruvate;

25 and

c) reacting pyruvate with acetolactate synthase enzyme under conditions appropriate to produce a compound that does not react with transaminase.

30 26. The process of Claim 25 wherein in step (a) the enzyme is deaminase enzyme.

27. The process of Claim 26 wherein deaminase enzyme is present in aqueous solution as whole cells producing  
35 deaminase enzyme.

28. The process of Claim 27 wherein the whole cells are present in aqueous solution at a cell load of about

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50 mg/ml to about 200 mg/ml.

29. The process of Claim 28 wherein the whole cells  
are present in aqueous solution at a cell load of about  
5 100 mg/ml.

30. The process of Claim 26 wherein deaminase enzyme  
is present in aqueous solution as cell extracts of  
whole cells producing deaminase enzyme.

10

31. The process of Claim 26 wherein deaminase enzyme  
is present as immobilized enzyme.

15

32. The process of Claim 26 wherein deaminase enzyme  
is produced by a cell from a non-naturally occurring  
microorganism comprising a gene that encodes for  
deaminase enzyme.

20

33. The process of Claim 32 wherein the cell comprises  
a plasmid that comprises a gene that encodes for  
deaminase enzyme.

25

34. The process of Claim 33 wherein the cell comprises  
as plasmid that comprises a gene that encodes for  
threonine deaminase enzyme.

35. The process of Claim 34 wherein the plasmid is  
pME64.

30

36. The process of Claim 25 wherein the compound that  
does not react with transaminase enzyme is  
acetolactate.

35

37. The process of Claim 25 wherein the compound that  
does not react with transaminase enzyme is acetoin.

38. The process of Claim 25 wherein the first amino  
acid is L-threonine.

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39. The process of Claim 25 wherein the second amino acid is alanine.

40. The process of Claim 25 wherein the second amino acid is L-aspartate.

41. The process of claim 25 wherein the keto acid is 2-ketobutyrate.

10 42. The process of Claim 25 wherein transaminase enzyme is present in aqueous solution as whole cells producing transaminase enzyme.

15 43. The process of Claim 42 wherein the whole cells are present in aqueous solution at a cell load of about 50 mg/ml to about 200 mg/ml.

20 44. The process of Claim 43 wherein the whole cells are present in aqueous solution at a cell load of about 100 mg/ml.

45. The process of Claim 25 wherein transaminase enzyme is present in aqueous solution as cell extracts of whole cells producing transaminase enzyme.

25

46. The process of Claim 25 wherein transaminase enzyme is present as immobilized enzyme.

30 47. The process of Claim 25 wherein transaminase enzyme is produced by a cell from a non-naturally occurring microorganism comprising a gene that encodes for transaminase enzyme.

35 48. The process of Claim 47 wherein the cell comprises a plasmid that comprises a gene that encodes for transaminase enzyme.

49. The process of Claim 48 wherein the plasmid is

pME64.

50. The process of Claim 25 wherein acetolactate synthase enzyme is present in aqueous solution as whole  
5 cells producing acetolactate synthase enzyme.

51. The process of Claim 50 wherein the whole cells are present in aqueous solution at a cell load of about 50 mg/ml to about 200 mg/ml.

10

52. The process of Claim 51 wherein the whole cells are present in aqueous solution at a cell load of about 100 mg/ml.

15

53. The process of Claim 25 wherein acetolactate synthase enzyme is present in aqueous solution as cell extracts of whole cells producing acetolactate synthase enzyme.

20

54. The process of Claim 25 wherein acetolactate synthase enzyme is present as immobilized enzyme.

25

55. The process of Claim 25 wherein acetolactate synthase enzyme is produced by a cell from a non-naturally occurring microorganism comprising a gene that encodes for acetolactate synthase enzyme.

30

56. The process of Claim 55 wherein the cell comprises a plasmid that comprises a gene that encodes for acetolactate synthase enzyme.

57. The process of Claim 56 wherein the plasmid is pPOT300.

35

58. The process of Claim 25 further comprising the step of isolating the third amino acid.

59. A process for making L-2-aminobutyrate comprising:

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- a) reacting L-threonine with threonine deaminase under conditions appropriate to produce 2-ketobutyrate;
- b) reacting the 2-ketobutyrate, L-aspartate and transaminase enzyme under conditions appropriate to 5 produce oxolactate and L-2-aminobutyrate;
- c) allowing the oxolactate to form pyruvate;
- d) reacting the pyruvate with acetolactate synthase enzyme under conditions appropriate to produce acetolactate;
- 10 e) allowing the acetolactate to form acetoin; and
- f) separately recovering acetoin and L-2-aminobutyrate.

60. Reaction medium comprising a keto acid, an amino 15 acid, transaminase enzyme, and acetolactate synthase enzyme.

61. The reaction medium of Claim 60 wherein the amino acid is alanine.

20

62. The reaction medium of Claim 60 wherein the amino acid is L-aspartate.

25

63. The reaction medium of claim 60 wherein the keto acid is 2-ketobutyrate.

64. The reaction medium of Claim 60 wherein transaminase enzyme is present in aqueous solution as whole cells producing transaminase enzyme.

30

65. The reaction medium of Claim 64 wherein the whole cells are present in aqueous solution at a cell load of about 50 mg/ml to about 200 mg/ml.

35

66. The reaction medium of Claim 65 wherein the whole cells are present in aqueous solution at a cell load of about 100 mg/ml.

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67. The reaction medium of Claim 60 wherein transaminase enzyme is present in aqueous solution as cell extracts of whole cells producing transaminase enzyme.

5

68. The reaction medium of Claim 60 wherein transaminase enzyme is present as immobilized enzyme.

69. The reaction medium of Claim 60 wherein  
10 transaminase enzyme is produced by a cell from a non-naturally occurring microorganism comprising a gene that encodes for transaminase enzyme.

R

70. The reaction medium of Claim 69 wherein the cell  
15 comprises a plasmid that comprises a gene that encodes for transaminase enzyme.

71. The reaction medium of Claim 70 wherein the plasmid is pME64.

20

72. The reaction medium of Claim 60 wherein acetolactate synthase enzyme is present in aqueous solution as whole cells producing acetolactate synthase enzyme.

25

73. The reaction medium of Claim 60 wherein the whole cells are present in aqueous solution at a cell load of about 50 mg/ml to about 200 mg/ml.

30 74. The reaction medium of Claim 73 wherein the whole cells are present in aqueous solution at a cell load of about 100 mg/ml.

35 75. The reaction medium of Claim 60 wherein acetolactate synthase enzyme is present in aqueous solution as cell extracts of whole cells producing acetolactate synthase enzyme.

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76. The reaction of Claim 60 wherein acetolactate synthase enzyme is present as immobilized enzyme.

77. The reaction medium of Claim 60 wherein  
5 acetolactate synthase enzyme is produced by a cell from  
a non-naturally occurring microorganism comprising a  
gene that encodes for acetolactate synthase enzyme.

78. The reaction medium of Claim 77 wherein the cell  
10 comprises a plasmid that comprises a gene that encodes  
for acetolactate synthase enzyme.

79. The reaction medium of Claim 77 wherein the  
plasmid is pPOT300.

15

80. The reaction medium of Claim 60 further comprising  
a deaminase.

81. The reaction medium of claim 80 further comprising  
20 L-threonine.

82. Plasmid pME64.

83. Plasmid pME64.

25

84. Plasmid pPOT300.

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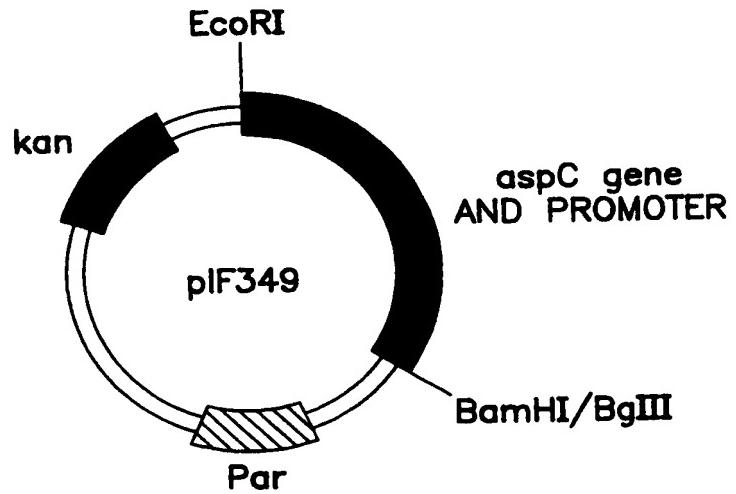
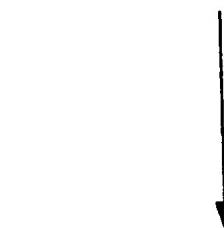
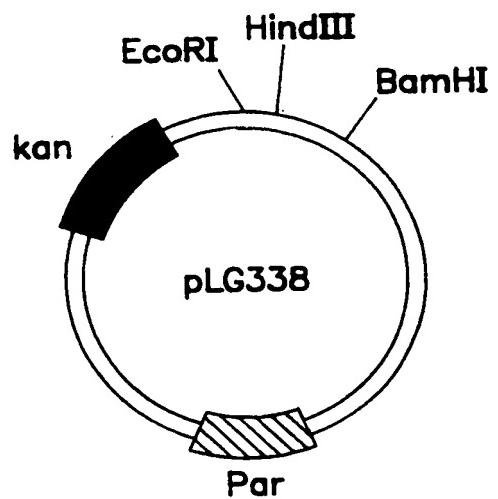


FIG. 1

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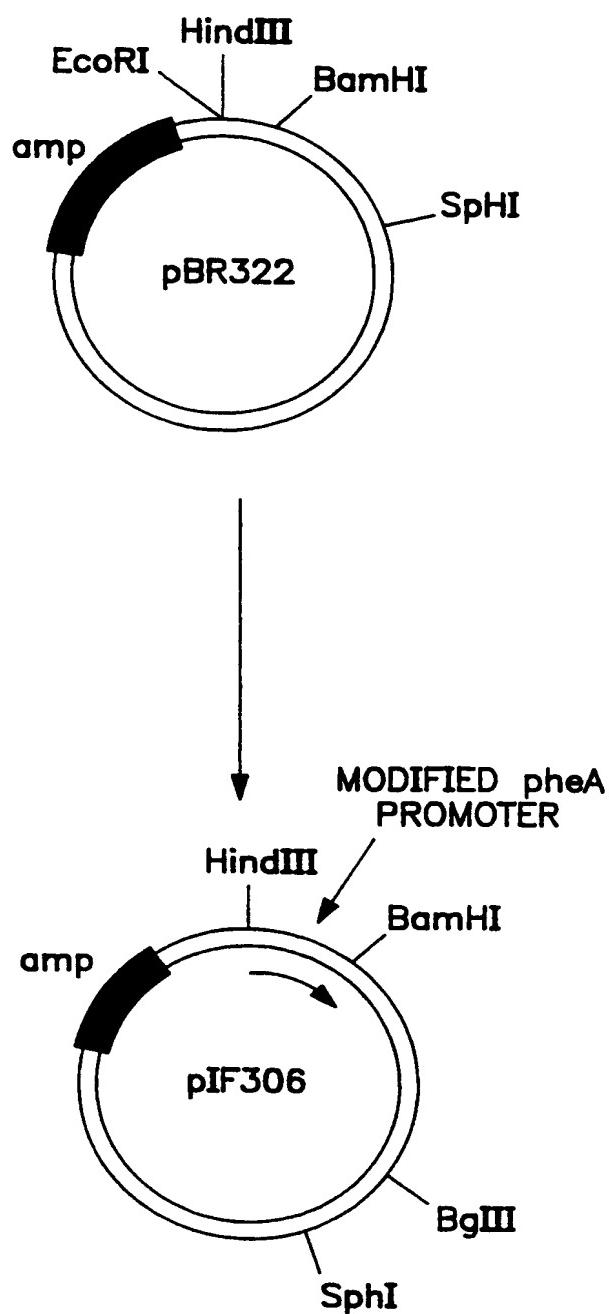


FIG. 2

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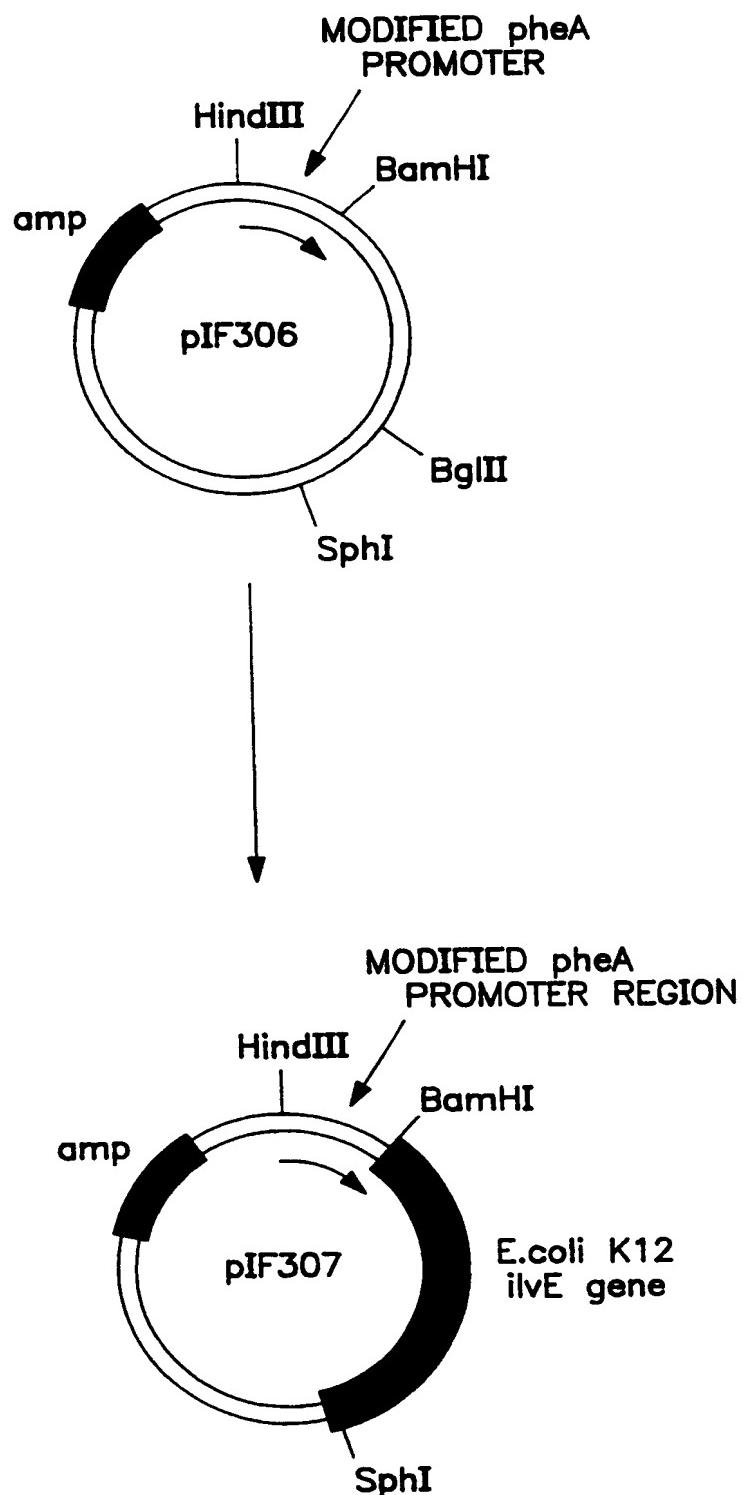


FIG. 3

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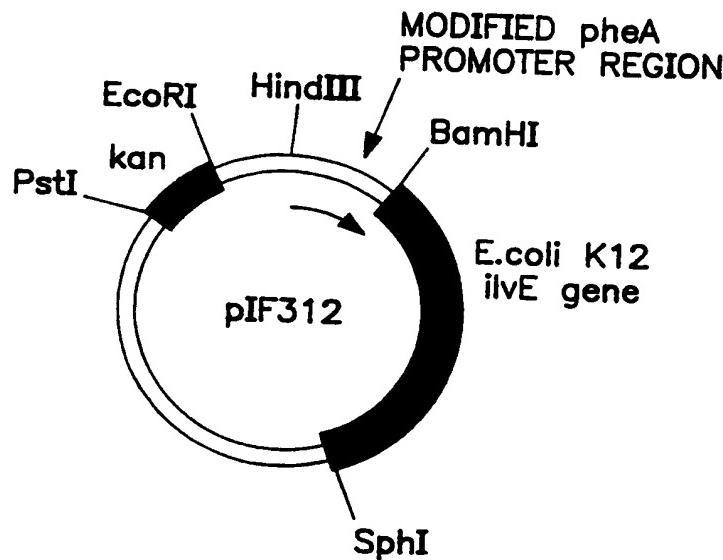
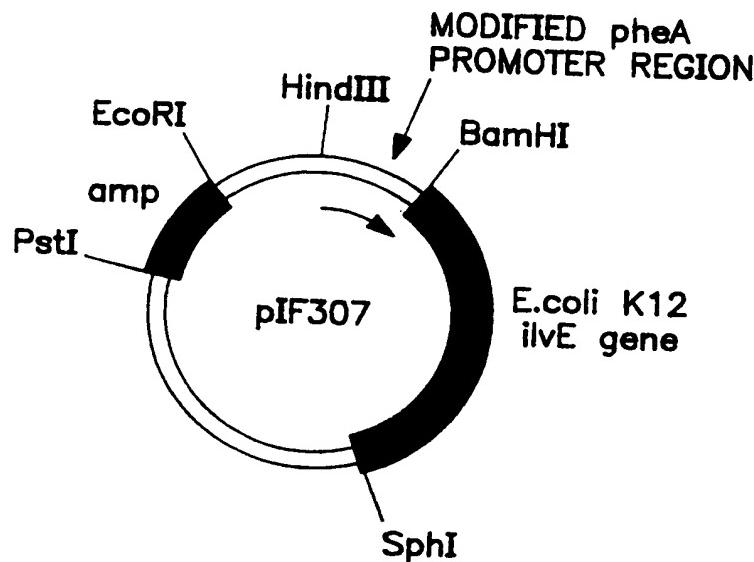


FIG. 4

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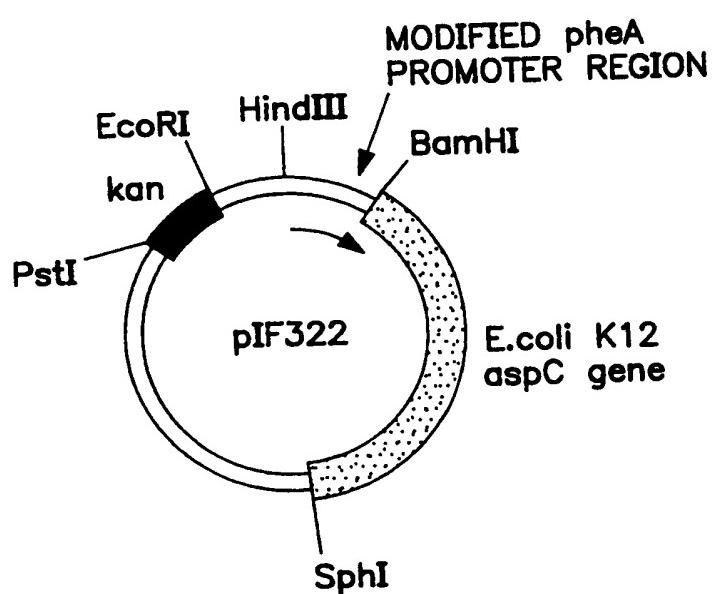
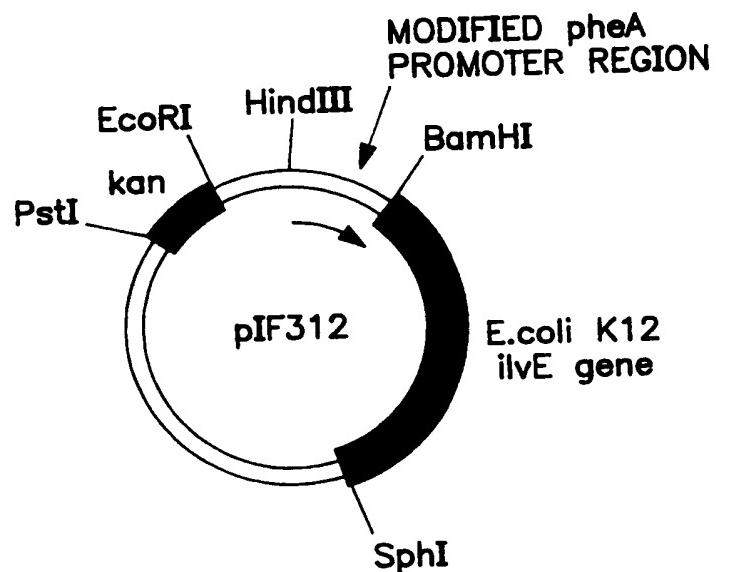


FIG. 5

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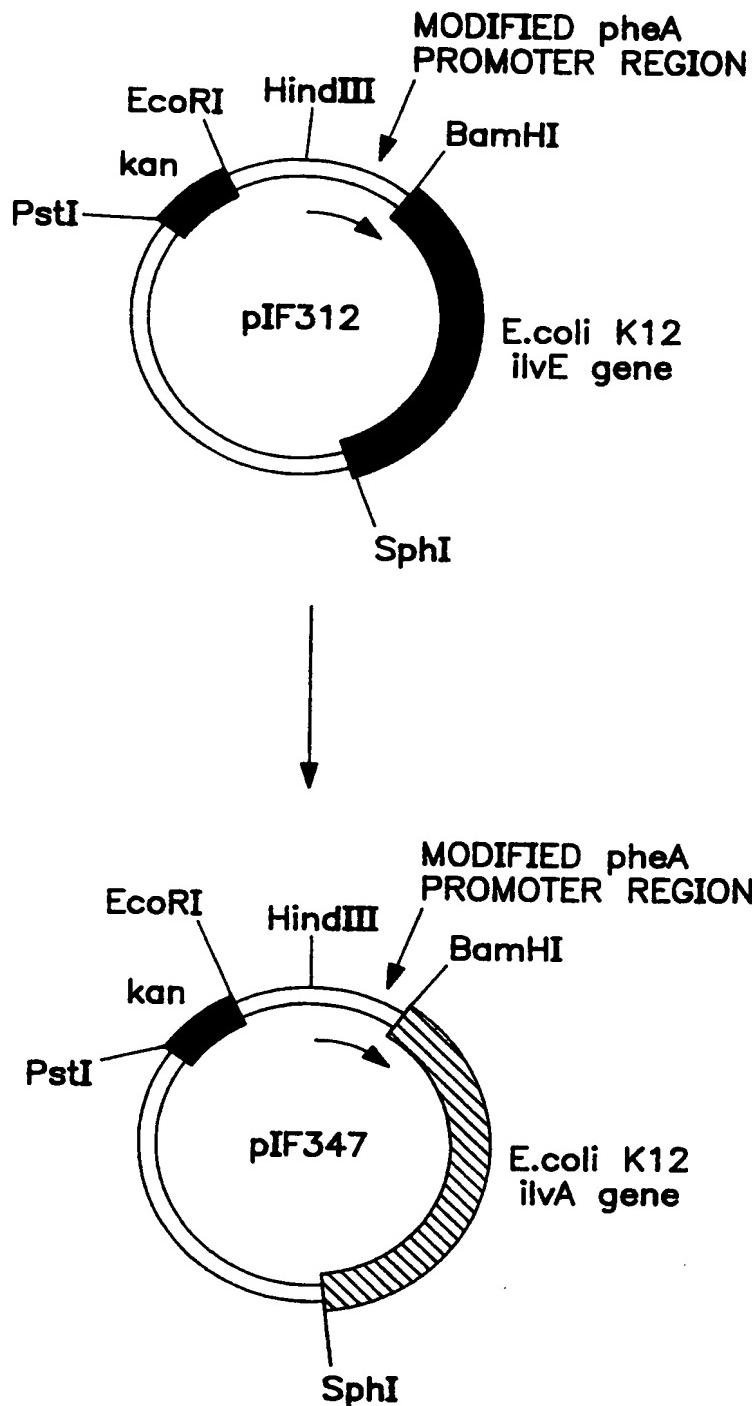


FIG. 6

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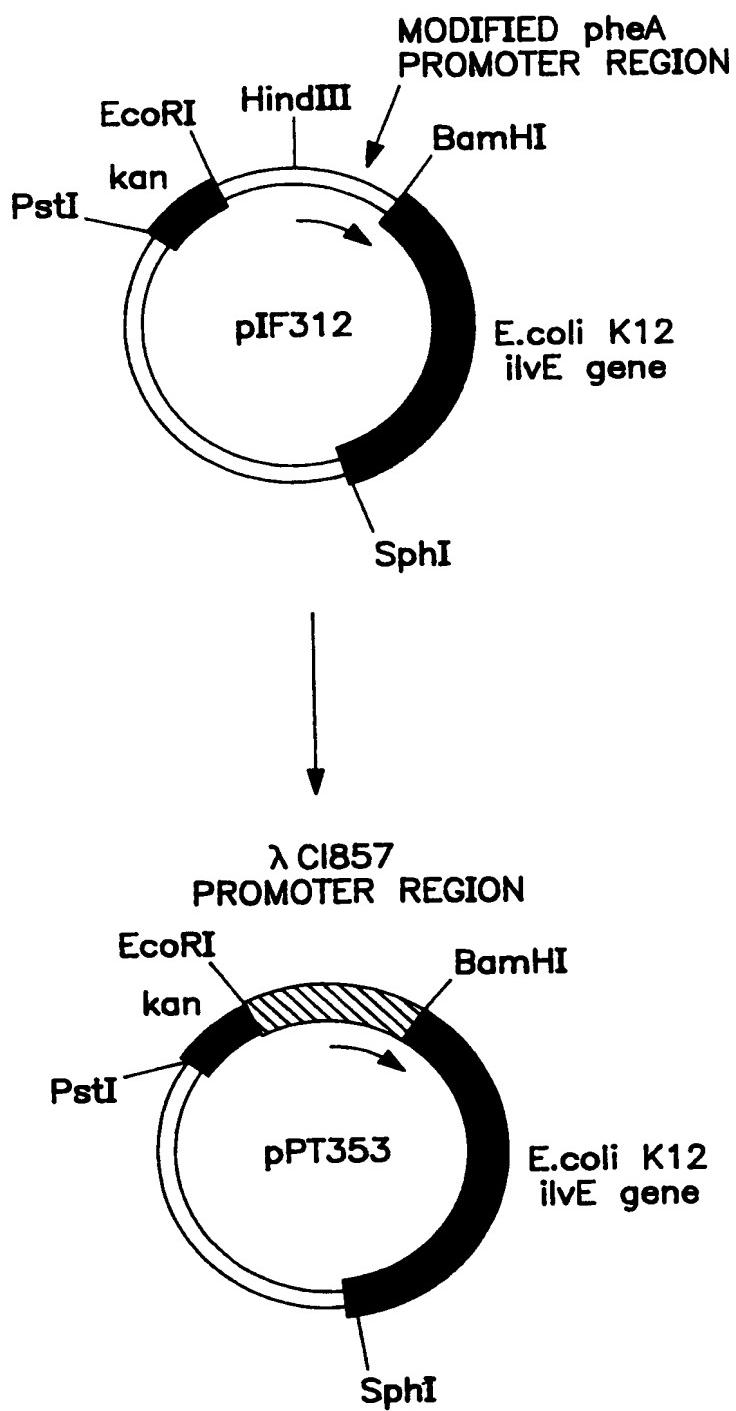


FIG. 7

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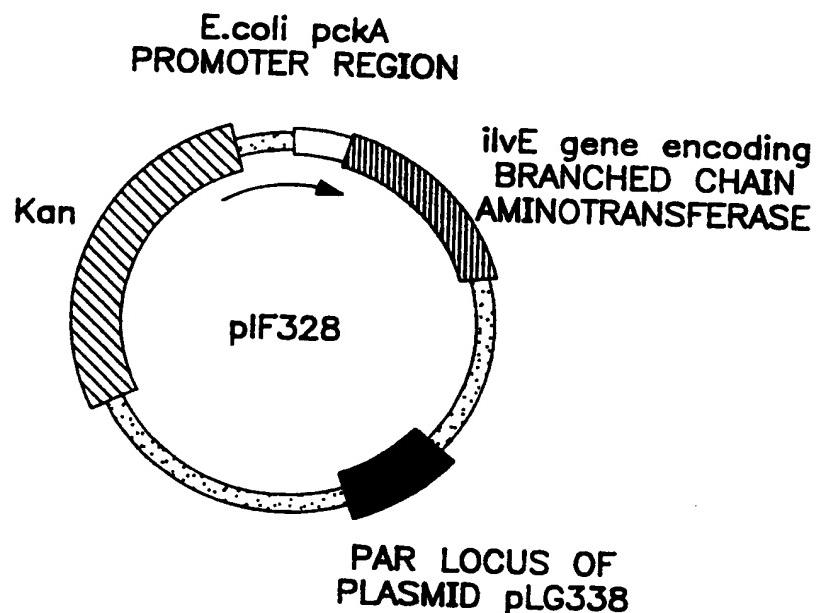


FIG. 8

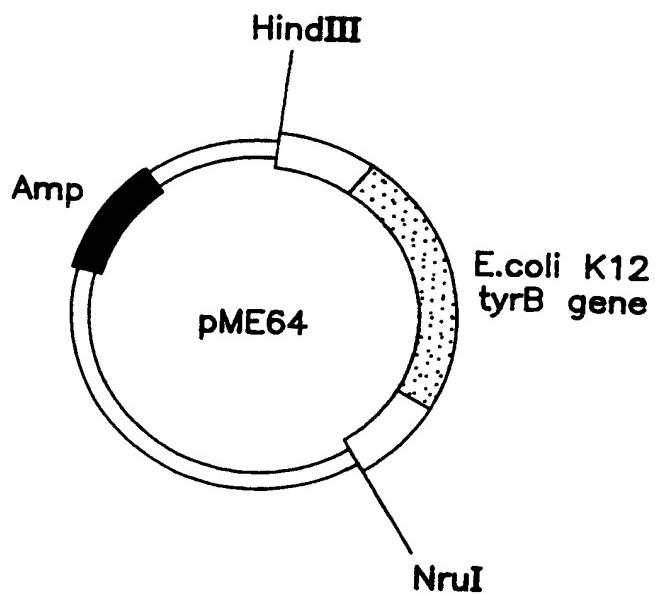


FIG. 9

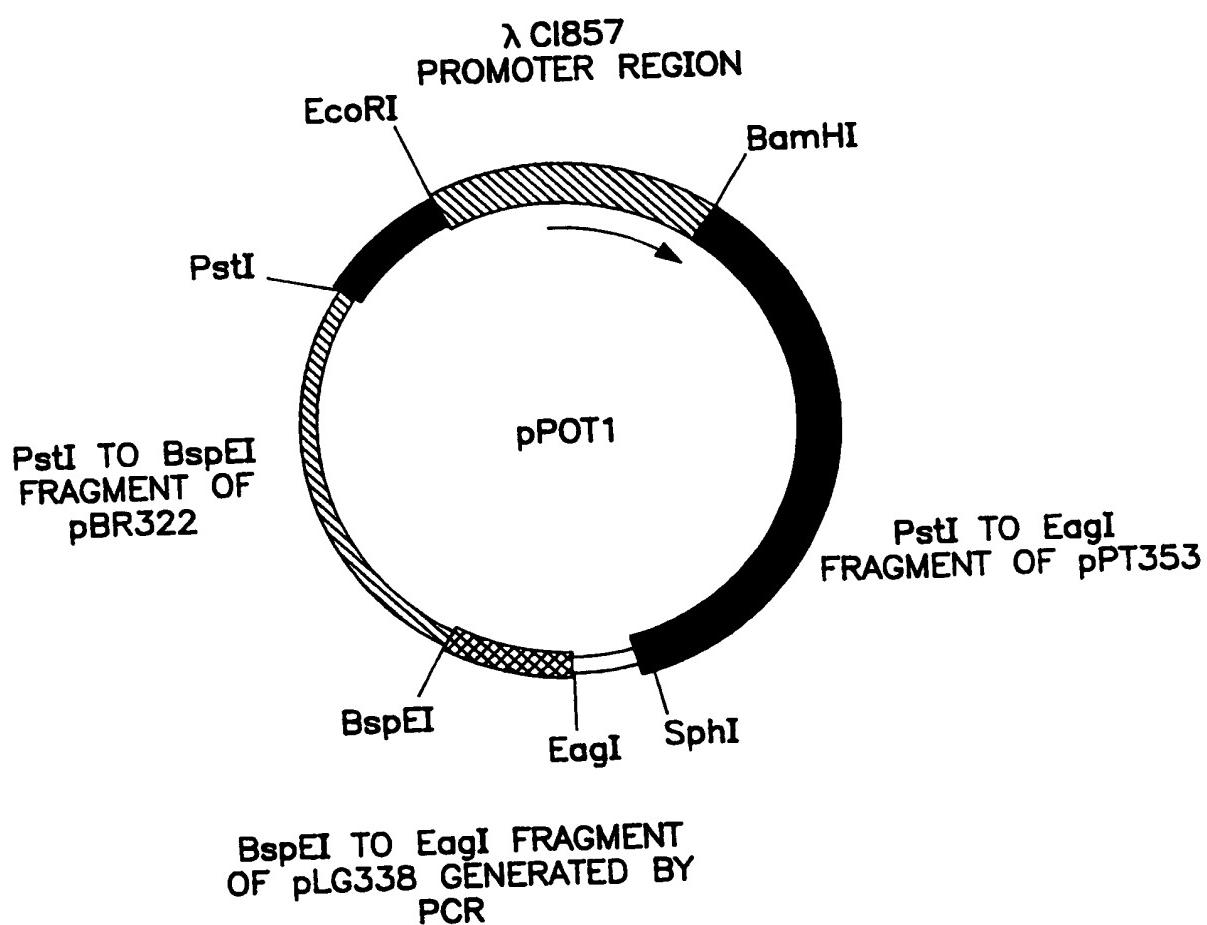
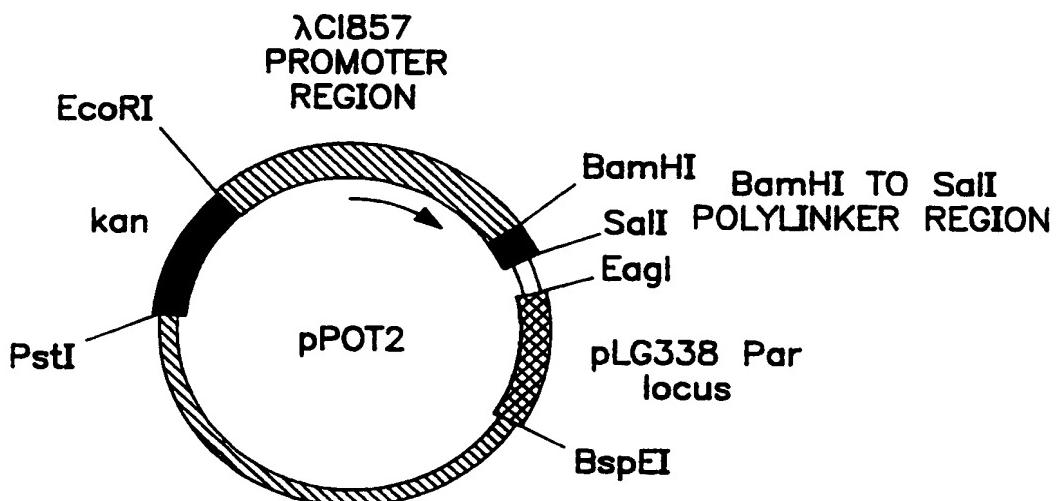
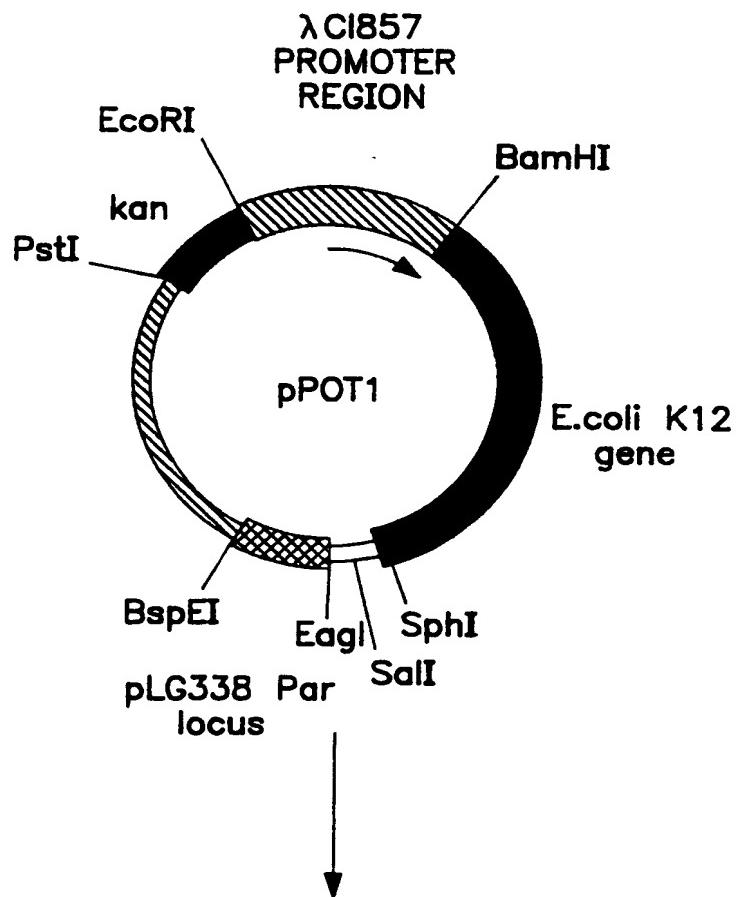


FIG. 10

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**FIG. 11**

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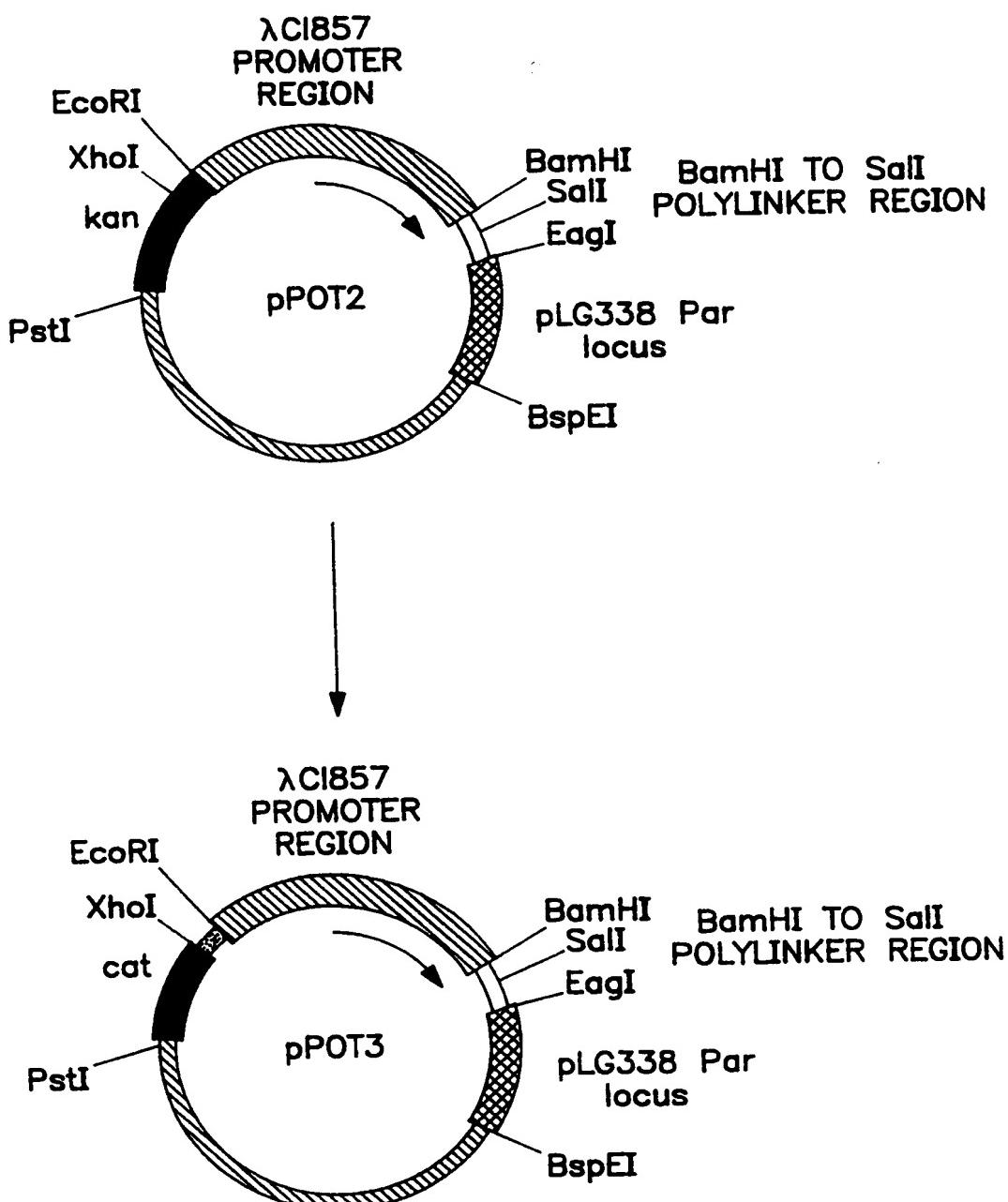


FIG. 12

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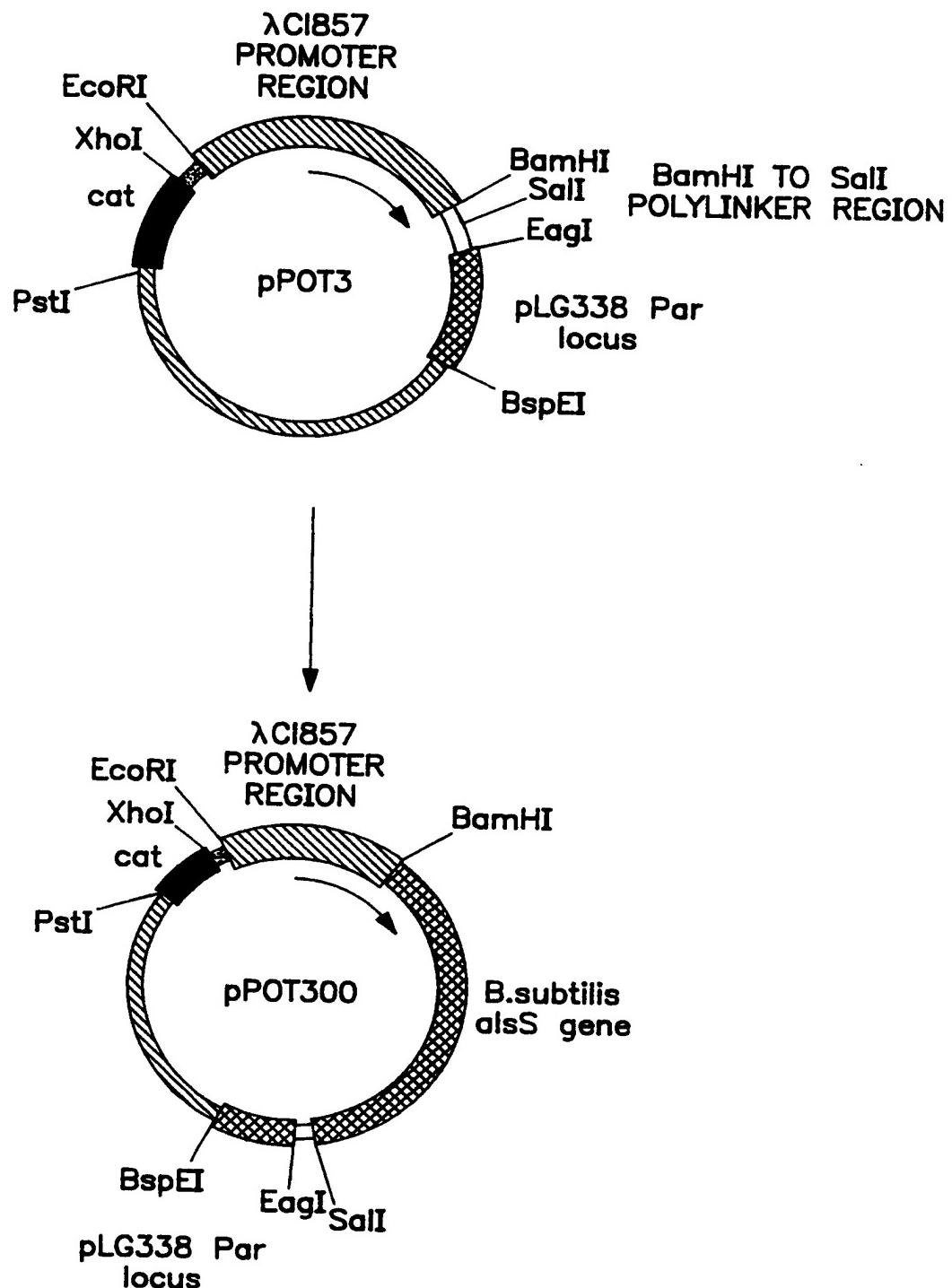


FIG. 13

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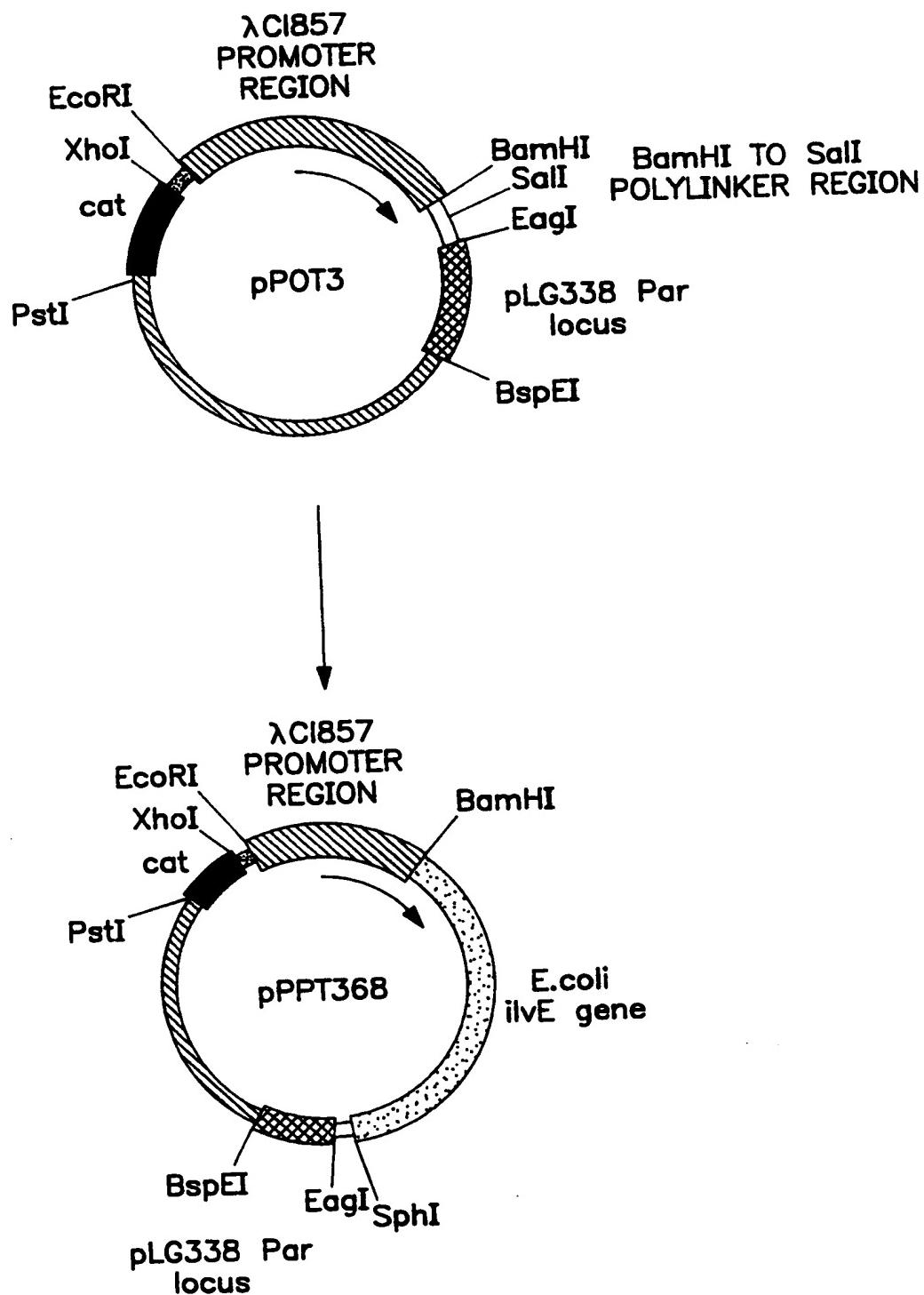


FIG. 14

# INTERNATIONAL SEARCH REPORT

In:	Int'l Application No PCT/US 98/10169
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<b>A. CLASSIFICATION OF SUBJECT MATTER</b>					
IPC 6 C12P13/04 C12P7/26 C12P7/62 C12N15/63 C12N9/88 C12N9/10					
According to International Patent Classification (IPC) or to both national classification and IPC					
<b>B. FIELDS SEARCHED</b>					
Minimum documentation searched (classification system followed by classification symbols)					
IPC 6 C12P C12N					
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched					
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)					
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>					
Category	Citation of document, with indication, where appropriate, of the relevant passages				Relevant to claim No.
X	US 5 316 943 A (G. E. KIDMAN ET AL.) 31 May 1994				82,83
Y	see the whole document				1-81,84
X	I. G. FOTHERINGHAM ET AL.: "The cloning and sequence analysis of the <i>aspC</i> and <i>tyrB</i> genes of <i>Escherichia coli</i> K12." <i>BIOCHEMICAL JOURNAL</i> , vol. 234, no. 3, 1986, pages 593-604, XP002078391 cited in the application				82,83
Y	see the whole document				1-81,84
					-/-
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.			<input checked="" type="checkbox"/> Patent family members are listed in annex.		
Special categories of cited documents :					
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"O" document referring to an oral disclosure, use, exhibition or other means			"Z" document member of the same patent family		
"P" document published prior to the international filing date but later than the priority date claimed					
Date of the actual completion of the international search			Date of mailing of the international search report		
23 September 1998			07/10/1998		
Name and mailing address of the ISA			Authorized officer		
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016			Douschan, K		

**INTERNATIONAL SEARCH REPORT**

Int'l Application No
PCT/US 98/10169

**C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. D. MARUGG ET AL.: "Identification and characterization of the alpha-acetolactate synthase gene from Lactococcus lactis subsp. lactis biovar diacetylactis." APPL. ENVIRON. MICROBIOL., vol. 60, no. 4, 1994, pages 1390-1394, XP002078392 see the whole document ----	1-81,84
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Int:	Application No
PCT/US 98/10169	

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